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Effect of Copper Ions on Thermal Stability of Glucoamylase from *Fusarium* sp.

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ABSTRACT

Fusarium solani, a mesophilic fungus, was grown for 96 h at 35°C, pH 5 under solid state growth conditions using wheat bran for the production of glucoamylase. The enzyme was purified using ammonium sulfate precipitation, anion-exchange chromatography and gel filtration. Addition of copper ions activated the enzyme. Thermal inactivation follows first order kinetics. The half-life of native enzyme was 24.75 min at 60°C while that of copper modified 57.76 min. Moreover, free energy (ΔG*) and enthalpy (ΔH*) of activation for thermal denaturation of copper modified glucoamylase were also high (106.40 k J mol^{-1} & 120.40 k J mol^{-1}) as compared to native. It is suggested that copper modified glucoamylase is highly thermostable and is suitable for industrial applications.

Key Words: Amyloglucosidase; Gamma amylase; Thermodynamics; Thermal denaturation; *Fusarium solani*

INTRODUCTION

Glucoamylase (synonym amyloglucosidase, starch glucogenase, exo-1, 4-α-D-glucan-glucanohydrolase, EC 3.2.1.3) belongs to the most important catalytically active proteins having broad possibilities of technical use. It is an exoacting enzyme that yields ß-D-glucose from the non-reducing chain ends of *amylose* and amylopectin by hydrolyzing α-1, 4 linkages in a stepwise manner (Fogarty, 1983; Marlida *et al.,* 2000). It also hydrolyses α-1, 6 and the rare α-1, 3 linkages although at much slower rate (Specka *et al.,* 1991).

Amylases are produced by various organisms, including bacteria, fungi and yeast. These enzymes have found wide applications in the processed food industry, fermentation industry, textile and paper industries (Selvakumar *et al*., 1996). Traditionally, glucoamylase has been produced by submerged fermentation (SmF). In recent years; however, the solid-state fermentation (SSF) processes have been increasingly applied for the production of this enzyme. Agro-industrial residues are generally considered the best substrates for SSF processes and enzyme production in SSF is not an exception to that.

Enzymes may require metal ions for their maximal catalytic activity and are termed as holoenzymes. Metals are responsible for right orientation of active site of holoenzymes. Metal binding to enzyme is one of the factors responsible for protein stabilization. The interaction between protein and metal ions and their effect on structure and function is difficult to study. The folded state of protein can be stabilized by metal binding during which metal ions are coordinated usually by lone pair donation from oxygen or nitrogen atoms. The contribution of metal binding to protein stability comes from the studies of Kellis *et al.* (1991) who introduced a metal chelating site into an alpha helix of iso-cytochrome c in the presence of saturating Cu (II).

Glucoamylase play an important role in the improvement of starch based food products. It is highly desirable to increase the thermal stability of glucoamylase so that starch hydrolysis could be carried out at higher temperature. Therefore, the present study was undertaken to study the effect of copper ions on the thermal stability of glucoamylase from *Fusarium solani.*

MATERIALS AND METHODS

Isolation and purification of enzyme. Pure culture of *Fusarium solani* was obtained from National Fungal Culture Collection of Pakistan (NFCCP), Department of Plant Pathology, University of Agriculture, Faisalabad. The culture was maintained on potato dextrose agar (PDA) slants. The fungus was grown on wheat bran under solid state fermentation. At the end of fermentation, the enzyme was extracted with distilled by squeezing the mixture through muslin cloth. The crude extract was then centrifuged at 10000 x g at 4°C for 30 min and the clear supernatant was used as the enzyme source. The enzyme was partially purified using ammonium sulfate precipitation, ion-exchange chromatography and gel filtration with 6.32-fold increase in the specific activity (23.08 to 145.79 U mg^{-1} protein) with 35.65% recover as described by Deutscher (1990).

Protein estimation. Total proteins were estimated by Bradford micro assay (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

Determination of glucoamylase activity. Glucoamylase activity was determined by using 1% soluble starch solution in 50 mM MES buffer (pH 5.5) at 40°C for 40 min. The

released glucose was measured using a glucose oxidase (GOD) method as described previously (Iqbal *et al*., 2003). One unit of glucoamylase activity was defined as the amount of enzyme required to liberate 1 µ mole of glucose equivalent ml^{-1} min^{-1} under the assay conditions".

Effect of Cu^{2+}. The glucoamylase from *Fusarium solani* was dialyzed against the assay buffer containing 10 mM EDTA to remove the metal ions. The enzyme was then dialyzed against the assay buffer to completely remove EDTA as reported by Siddiqui *et al*. (1997). The type of glucoamylase activation/inhibition by Cu^{2+} was determined at 1 to 10 mM concentration of Cu^{2+}. Maximum activity was observed at 5 mM concentration.

Thermal stability. Thermal inactivation of native and Cu^{2+} modified glucoamylases were determined by incubating enzymes in 50 mM acetate buffer having pH 5.5 at different temperatures. Time course aliquots withdrawn, cooled on ice for at least 30 minutes and then assayed for glucoamylase activity at 40°C at described before (Rashid & Siddiqui, 1998). This procedure was repeated at four different temperatures (50, 54, 57 & 60°C). The data was fitted to first order plots and analyzed as described by Munch and Tritsch (1990) and Montes *et al.* (1995). The first-order rate constants for irreversible thermal denaturation (K_d) of glucoamylases were determined and Arrhenius plots were applied to determine the activation energy for denaturation (Ea).

The thermodynamic parameters for thermostability were calculated by rearranging the Eyring's absolute rate equation derived from the transition state theory (Eyring & Stearn, 1939) as described by Siddiqui *et al.* (1999).

$$K_d = (k_b T/h)\, e^{(-\Delta H^*/RT)} .e^{(\Delta S^*/R)} \quad (1)$$

Where, h = Planck's constant = 6.63×10^{-34} Js

k_b = Boltzman's constant (R/N) = 1.38×10^{-23} Jk^{-1}

R = gas constant = 8.314 Jk^{-1} mol^{-1}

N = Avogadro's No. = 6.02×10^{23} mol^{-1}

T = Absolute temperature

$$\Delta H^* = Ea - RT \quad (2)$$

$$\Delta G^* = RT \ln (K_d, h/k_b. T) \quad (3)$$

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T \quad (4)$$

RESULTS AND DISCUSSION

Thermal denaturation of enzymes occurs in two steps as shown below:

$$N \leftrightarrow U \rightarrow I$$

Where N is the native enzyme, U is the unfolded inactive enzyme which could be reversibly refolded upon cooling and I is the inactivated enzyme formed after prolong exposure to heat and therefore cannot be recovered upon cooling. The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (Daniel, 1996). The opening up of enzyme structure is accompanied by an increase in disorder or entropy of activation (Vieille & Zeikus, 1996).

The kinetics and thermodynamics of irreversible thermal denaturation of native and copper modified glucoamylases from *Fusarium solani* were studied at 50 to 60°C. Pseudo first order plots were applied to determine the extent of thermal inactivation. The results regarding the thermal inactivation of native and copper modified glucoamylases are shown in (Table I & II). It is obvious from the results that coupling of copper with glucoamylase resulted into stable enzyme. Native glucoamylase was stable up to 50°C and showed a half-life of 74.53 min (Table I). With increase in temperature the half-life decreases. At 60°C, the native form displayed a half-life of 24.75 min only indicating that the enzyme is not thermostable at higher temperatures. While the copper modified form displayed a half-life of 57.76 min indicating that the modified form was stable at higher temperature (Table II).

In order to determine the thermodynamic parameters for irreversible thermal stability, the energy of activation for thermal denaturation was determined by applying the Arrhenius plots (Figs. 1 & 2). The Gibbs free energy (ΔG^*) for thermal unfolding at 50°C for native glucoamylase was 103.09 k $Jmol^{-1}$ (Table I & II) while that of copper modified 106.34 k $Jmol^{-1}$.With increase in temperature free energy decreases but copper modified form still possessed high free energy for thermal unfolding (105.44 k $Jmol^{-1}$).

The enthalpy of thermal unfolding (ΔH^*) of native form was 98.66 k $Jmol^{-1}$ at 50 °C while that of modified form 120.39 k $Jmol^{-1}$ which indicated that high energy is required for thermal denaturation of enzyme after copper

Table I. Kinetic and thermodynamic parameters for irreversible thermal denaturation of native glucoamylase from *Fusarium solani*

Temp. (°C)	K_d (min^{-1})	$t_{½}$ (min)	ΔH^* ($kJmol^{-1}$)	ΔG^* ($kJmol^{-1}$)	ΔS^* ($Jmol^{-1}K^{-1}$)
50	0.009	74.53	98.66	103.09	-13.05
53	0.015	46.21	98.63	102.87	-9.144
56	0.023	30.13	98.61	102.36	-11.42
60	0.028	24.75	98.57	101.61	-13.57

Where K_d (first order rate constant of denaturation) is determined from Fig.1

$t_{½}$ (half-life) = $\ln 2/K_d$

Table II. Kinetic and thermodynamic parameters for irreversible thermal denaturation of glucoamylase in the presence of Cu^{+2}

Temp. (°C)	K_d (min^{-1})	$t_{½}$ (min)	ΔH^* ($kJmol^{-1}$)	ΔG^* ($kJmol^{-1}$)	ΔS^* ($Jmol^{-1}K^{-1}$)
50	0.003	225.70	120.39	106.34	45.02
53	0.004	172.42	120.37	106.29	43.68
56	0.005	129.56	120.34	105.85	42.53
60	0.012	57.76	120.31	105.44	44.66

Where K_d (first order rate constant of denaturation) is determined from Fig.2; $t_{1/2}$ (half- life) = $\ln 2/K_d$

Fig. 1. Arrhenius plot for determination of energy of activation for thermal denaturation of native glucoamylase

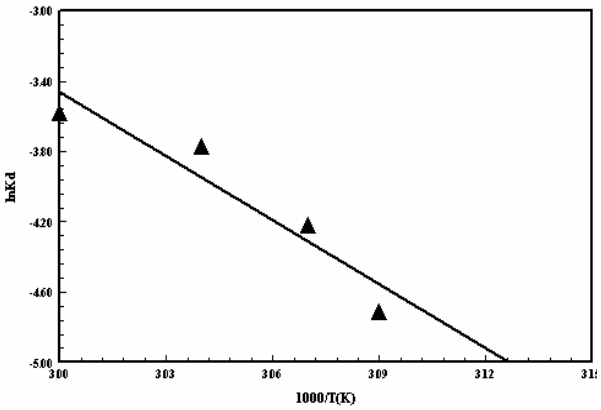
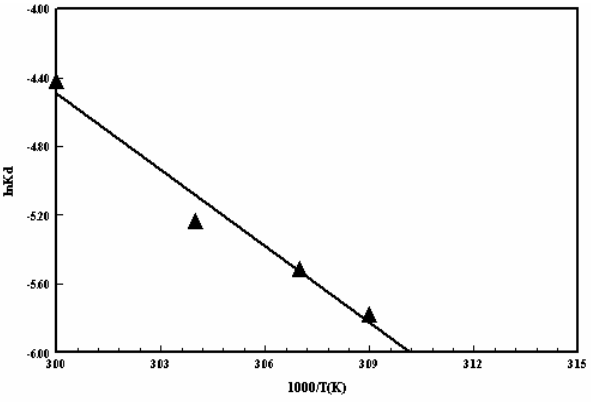


Fig. 2. Arrhenius plot for determination of activation energy for thermal denaturation of copper modified glucoamylase



coupling. Its value decreases with rise in temperature which revealed that less energy is required to denature enzyme at high temperature. The unfolding of enzyme structure is accompanied by an increase in disorder or entropy of activation, but contrary to this glucoamylase from *Fusarium solani* has negative entropy (ΔS*) which revealed that native form is in more ordered state. After coupling to copper the entropy became positive (45.02 J mol^{-1} K^{-1}).

The denaturation of many protein like chicken egg albumin (Eyring & Stearn, 1939) and lactoglobulin at low temperature and in the presence of urea show negative ΔS*, because water ordering increase in the vicinity of non-polar amino acids which are exposed during unfolding (Privalov & Gill, 1988). This ordering of water around hydrophobic residues is disrupted at higher temperature; therefore, this could not be the reason for negative ΔS* in case of native glucoamylase.

The unfolding of hemoglobin in acids at 45°C also showed negative ΔS* of -155 $Jmole^{-1}$ K^{-1} in the presence of acid (Eyring & Stearn, 1939), whereas, the thermal denaturation of myoglobin at 40°C and high pressure gave ΔS* of -84 $Jmole^{-1}$ K^{-1}).

CONCLUSION

In the light of above kinetic and thermodynamic data, it may be concluded that binding of Cu^{+2} thermostabilized the glucoamylase. Therefore, enzymes may be made thermostable in vitro by the attachment of Cu^{+2}.

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